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# Infected Cell in Trouble: Bystander Cells Ring the Bell

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**Infection with intracellular pathogens triggers cytokine production in the infected cells. In this issue of *Immunity,* Kasper et al. (2010) demonstrate that in certain infections, much of the response is mounted by noninfected neighboring cells.**

The epithelial surface is a key portal of entry for pathogens. However, invading pathogens are detected by pattern recognition receptors (PRRs), which activate the appropriate innate defenses (Ting et al., 2010). Depending on the type and the context of stimulation, sensing of the pathogen could trigger the activation of inflammasome, which facilitates maturation and secretion of preformed interleukin (IL)-1 family members and/or the expression of antimicrobial compounds, chemokines, and cytokines such as IL-8. The chemoattractants recruit phagocytes to the site of infection to facilitate pathogen elimination and initiate adaptive immune responses.

The mucosal epithelium was regarded as a passive element, contributing to host defense mainly by forming an impermeable, tight-junction-fortified monolayer prohibiting microbial access to the underlying tissue. This view has changed over the past decade as it became clear that epithelial cells express PRRs, including the Nod-like receptors Nod1 and Nod2, and that these PRRs recognize microbial products leading to the secretion of proinflammatory cytokines (Artis, 2008; Ting et al., 2010). Thus, infected epithelial cells produce key cytokines (e.g., IL-8, CXCL-2, TNF-α, GM-CSF), attract phagocytic cells, and ultimately orchestrate innate immune defense and initiate adaptive immunity (Eckmann and Kagnoff, 2005). Therefore, epithelia do play an active role in coordinating defense.

Based on classical receptor-response mechanisms, it has been assumed that the proinflammatory cytokines are produced by the infected cell itself. This made sense as each epithelial cell harbored the PRR and the signaling cascades for driving cytokine expression. However, in this issue of *Immunity*, Kasper et al. (2010) demonstrate a substantial contribution of the response came from the noninfected bystander cells. This study employed the invasive pathogen *Shigella flexneri* to study signaling of the NF-κB transcription factor pathway, as well as JNK, ERK, and p38 kinase activation and IL-8 secretion at the single cell level. Upon invasion, *S. flexneri* enters the host cell's cytosol, where it is recognized by Nod1, activating NF-κB, JNK-, and ERK and p38 signaling and inducing IL-8 secretion (Figure 1). Surprisingly, NF-κB, JNK, ERK, and p38 signaling and IL-8 secretion occurred not only in the infected cell itself but also in the surrounding, noninfected bystander cells. Notably, bystander activation occurred by 30 min of infection and the cytokine release by the bystander cells was even more pronounced than that of the infected cell itself. Therefore, the bystander cells amplified the defense signal within the infected epithelium.

Bystander cell activation has also been identified in a parallel study of the Gram-positive pathogen *Listeria monocytogenes* (Dolowschiak et al., 2010). Soon after this pathogen has reached the cytosol of murine intestinal $mIC_{cl2}$ cells, the noninfected bystander cells begin expressing the chemokines CXCL5 and CXCL2, a murine IL-8 homolog (Figure 1). Notably, both studies excluded the possibility that bystander cells are activated by paracrine signaling, e.g., by cytokines released from the infected cells. Thus, bystander activation is a new mechanism of general importance for defense against various intracellular pathogens.

It seems surprising that bystander cell activation has not been observed earlier. This is most likely linked to the types of reagents and assays generally used in the field. Most studies of innate immunity have employed purified pathogen-derived molecules, an approach prohibiting differential analysis of direct and indirect activation. Single cell analysis of bacterial infection has circumvented this technical problem.

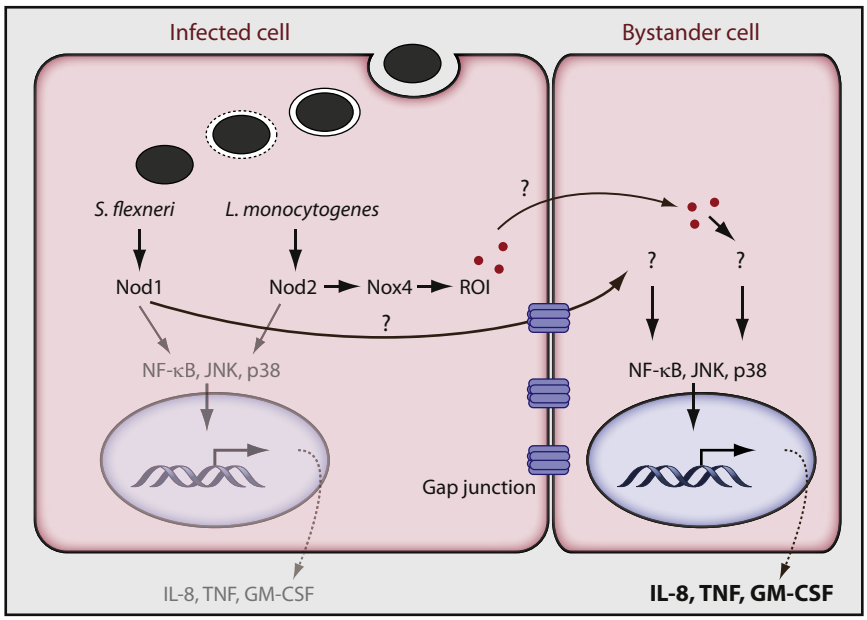


**Figure 1. Bystander Activation Drives Epithelial Cytokine Responses**
*Shigella* and *Listeria* spp. enter the host cell cytosol where cell wall components activate Nod1 or Nod2. *S. flexneri* thereby elicits (unidentified) molecular signals, which are transmitted to noninfected bystander cells via gap junctions. *L. monocytogenes* activates Nod2 and thereby Nox4, thus eliciting ROI, which are sensed (in an unknown fashion) by the bystander cell. In both cases, bystander cells respond by NF-κB-, JNK-, and p38-mediated cytokine production. Hence, the bulk of the cytokines are produced by noninfected bystander cells.

Pathogen mutants deficient in cell-to-cell spread and fluorescence techniques for detecting infected cells, noninfected cells, and host cellular activation were essential for discovering bystander activation.

The mechanism of signal transmission to the bystander cells is clearly of great interest and may reshape current mechanistic models of the cell biology of the innate immune response. Here, the two studies offer somewhat divergent explanations. In *S. flexneri*-infected human cells, the signal was elicited by the PRR Nod1 and required gap junctions, small water filled pores connecting neighboring epithelial cells (Kasper et al., 2010). Lack of expression or inhibition of gap junctions crippled bystander activation. However, the molecular nature of the activating signal transmitted through the gap junctions has not yet been identified (Figure 1). In contrast, *L. monocytogenes* required Nod2 for eliciting responses in murine $mIC_{cl2}$ cells and bystander activation was insensitive to gap junction inhibitors (Dolowschiak et al., 2010). Instead, bystander activation depended on reactive oxygen intermediates (ROIs; Figure 1). ROIs were produced by the epithelium-encoded NADPH oxidase Nox4. These ROIs were then detected in bystander cells, and ablation of ROI signaling by chemical inhibitors or siRNAs abolished bystander activation. Possibly, the differences in the proposed mechanisms are explained by the different nature of the host cells and of the pathogens which were investigated. However, it is tempting to speculate that different mechanisms of bystander cell activation may exist that might be triggered by different PRR. These will be important topics for future research.

What are the potential benefits of bystander cell activation? Clearly, signal amplification by elevating the total cytokine output per infection event represents one plausible advantage. Alternatively, bystander cell activation might represent a "safeguard feature" ensuring a proper defense, even if signaling in the infected cell is subverted by an invading pathogen. In fact, this has been demonstrated elegantly for *S. flexneri* (Kasper et al., 2010). This pathogen employs a type III secretion system not only for triggering epithelial cell invasion, but also for injecting the virulence factor OspF into the cytosol of the infected cell. OspF is a potent inhibitor of JNK, ERK and p38 signaling, resulting in the inhibition of proinflammatory gene expression by the infected cell (Li et al., 2007). Kasper et al. (2010) could show that this effect is restricted to the infected cell, whereas bystander cell responses remained unaffected. Moreover, IL-8 induction by a *S. flexneri ospF* mutant was equivalent in the infected and in the bystander cells. Thus, bystander activation seems to ensure defense even if cytokine production of the infected cell is inhibited by the pathogen. As pointed out by the authors (Kasper et al., 2010), this provides a plausible explanation for the puzzling observation that several mucosal pathogens which elicit pronounced mucosal inflammation, do express potent inhibitors of NF-κB, JNK, ERK, and p38 signaling, e.g., OspF and OspG from *S. flexneri* or AvrA from *Salmonella typhimurium* (Jones et al., 2008; Kim et al., 2005).

Are there virulence factors subverting bystander cell activation? In the case of *S. flexneri*, bystander cell activation occurred with very high efficiency. In contrast, *L. monocytogenes* and *S. typhimurium* were slightly less effective (Kasper et al., 2010). The reasons for this are currently unclear. However, one may speculate that virulence factors dampening bystander cell activation (via gap junctions and/or ROI) might be involved. Stealthy invasive pathogens like *Chlamydia* spp. or *Salmonella typhi*, which can invade epithelia without triggering pronounced responses, might be a promising source of such virulence factors. Again, this is purely speculative. But the identification of bystander cell activation has opened the door for addressing this question.

So far, bystander cell activation has been studied in tissue culture. It will be important to probe its role in vivo during the course of infection. PRR signaling, which seems to be central to bystander cell activation, is subject to regulation. Desensitization of PRR responses has been observed upon continued exposure to microbial products (Foster et al., 2007; Lotz et al., 2006). Similarly, bystander activation might be downregulated in the gut or other microbe-exposed epithelia. In this case, erroneous regulation of bystander activation might play a role in the genesis of some inflammatory bowel diseases. In addition, bystander activation may occur in deeper tissues that are normally not exposed to microbes. This would be in line with the observation of bystander

activation in mouse embryonic fibroblasts and human umbilical vein endothelial cells (Kasper et al., 2010). Systematic studies of bystander activation in different infected tissues will be of significant interest for understanding bacterial pathogenesis and host response to infection.

In conclusion, bystander cell activation may shift the paradigm of epithelial cell function in infectious disease. This mechanism transforms our view of the innate immune response and may have a broad relevance for innate immune defense against intracellular pathogens. The direct communication between infected and noninfected cells of an infected tissue raises numerous questions for cell biology, immunology, and bacterial pathogenesis.

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# Trapped versus Soluble Chemokines: Functions in Leukocyte Adhesion and Motility

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**In this issue of *Immunity*, Bao et al. (2010) provide in vivo evidence that heparan sulfate glycosaminoglycans (GAGs) are indispensable for immobilization and function of major chemokines required for leukocyte adhesion to and crossing through blood and lymphatic vessels.**

Chemokines are structurally related chemotactic cytokines (chemoattractants) with remarkable functional versatility (Bromley et al., 2008). Chemokines signal through cognate G protein coupled receptors (GPCRs) either at their soluble or immobilized states. However, a direct in vivo proof for their functions in soluble versus immobilized states has been difficult to obtain, because soluble chemokines are readily removed by conventional histological analysis. Thus, a genetic interference with chemokine immobilization has been necessary in order to dissect the significance of immobilization for particular adhesive and migratory processes.

Leukocyte extravasation from blood involves sequential chemoattractant-mediated signals. Chemokines stably immobilized on surface proteoglycans on the luminal surface of endothelial cells were suggested to play a pivotal role in integrin-mediated arrest of rolling leukocytes (Ley et al., 2007). Additional chemokine signals were suggested to promote crawling of leukocytes across endothelium, protrusion, and encounter of abluminal chemokines (Ley et al., 2007). Chemokine immobilization on vascular endothelial cells and adherent platelets was suggested to be critical not only to prevent their dilution by blood flow but also to facilitate localized signaling to integrins on rolling leukocytes. In addition, stroma-immobilized chemokines efficiently promote motility of lymphocytes and dendritic cells (DCs) in specific areas of lymphoid tissues (Bajenoff et al., 2006). However, it is still unclear whether chemokines that direct leukocyte motility and chemotaxis in various interstitial spaces as well as across epithelial barriers operate in their soluble or immobilized states (Schumann et al., 2010).

Most chemokines share a carboxyl terminus stretch of positively charged residues that recognize heparan sulfate (HS) GAGs with moderate affinities (Proudfoot, 2006; Rot and von Andrian, 2004). HS GAGs are ubiquitous and structurally diverse macromolecules that interact with many cytokines, growth factors, and extracellular matrix (ECM) components. In vitro and in vivo studies on leukocyte interactions with various endothelial cells have suggested that many chemokines immobilize, and at times also oligomerize on, HS GAGs (Proudfoot, 2006). The first in vivo involvement of endothelial heparan sulfate in inflammation was genetically supported by elegant endothelial-targeted ablation of the enzyme required for N-sulfation of HS GAGs (Wang et al., 2005). Attenuated neutrophil infiltration to sites of inflammation was reported in these mice but was attributed to combined inhibition of chemokine transcytosis across endothelial cells,